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EFFECT OF Ca^{+2} AND CHOLESTEROL ON ANTHOCYANIN FORMATION IN TURNIP SEEDLINGS

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ABSTRACT

The promotion of anthocyanin synthesis in turnip seedlings by five min exposure to red (R) light is inhibited by subsequent application of $CaCl_2$. The stimulation of dark synthesis of anthocyanin by n-propanol or by kinetin is also reduced by Ca^{+2} and by cholesterol, both of which are well known to stabilize cell membranes. By contrast, EDTA, which chelates Ca^{+2}, promotes dark synthesis of anthocyanin. Assay of native Ca^{+2} extractable from seedlings immersed in EDTA demonstrates that R light exposure promotes a highly significant increase in extractable Ca^{+2}.

KEY WORDS: Physiology, *Brassica*, anthocyanin, light response.

INTRODUCTION

It is well known that anthocyanin synthesis is promoted by light. In *Celosia* seedlings (Malaviya & Laloraya 1966) and buckwheat hypocotyl (Troyer 1964), anthocyanin synthesis is light dependent. Pecket & Hathout (1974a) suggested, from work on the low energy red (R)/far red (FR) reversible control of anthocyanin biosynthesis, that the locus of phytochrome action is in a membrane. Evidence for this view came from the fact that reagents such as n-propanol, which are believed to increase membrane permeability, cause a stimulation of dark synthesis of anthocyanin in red cabbage which can be nullified by exposure to far red light (Pecket & Hathout 1974b). It was suggested that the molecular configuration of phytochrome controls the passage of a substrate through a membrane to the site of anthocyanin biosynthesis. Further evidence for this view was provided by the fact that exogenously applied phenolic precursors, such as shikimic acid, promote dark synthesis of anthocyanin much more markedly after treatment of the tissue with propanol. Evidence has also been presented that kinetin, or red light, promotes anthocyanin synthesis in dark grown red cabbage and that its effect can be reversed by far

red light (Pecket & Hathout 1974a). Pecket & Small (1980), studied the site of anthocyanin synthesis in red cabbage seedling and reached the conclusion that the organelles responsible for anthocyanin formation (anthocyanoplasts) are found in the vacuoles of the plant cells and are the site of anthocyanin biosynthesis. Oelmuller & Mohr (1985), found that anthocyanin formation in milo seedlings occurs only in white and blue light, while red light and far red light are totally ineffective.

If the effects of light, kinetin and reagents such as propanol are indeed upon membrane permeability, then it would be expected that reversals of their effects should be brought about by the application of reagents such as calcium chloride (Davson 1951; Burstrom 1952), and cholesterol (Grunwald 1968), which are well known to act as membrane stabilizers. The work reported here concerns further investigation of the membrane based, phytochrome controlled, anthocyanin synthesis as evidenced by the effects of these membrane stabilizing agents.

MATERIALS AND METHODS

Seeds of turnip (*Brassica rapa*) provided from the Ministry of Agriculture were sown in Petri dishes, each having 25 seeds on two filter papers, moistened with 20 ml distilled water. They were germinated in the dark at 25° C.

Light sources.

Red light exposures were carried out in a cabinet illuminated by 4 fluorescent lamps, employing two sheets of red cinemoid No. 14 and one sheet of orange cinemoid No. 5.

Far red (FR) exposures were given in a cabinet illuminated by 4 fluorescent lamps and a filter system comprising two sheets of blue cinemoid No. 20 and two of orange No. 5, with a water screen between the lights and filters. Both chambers were placed in a dark room and all manipulations of material were carried out under a green safe light.

Anthocyanin extraction, assay and chromatography.

The anthocyanin in samples of 25 seedlings was completely extracted using 10 ml of 1% HCl in three successive aliquots of 5, 3 and 2 ml (Murave'va & Bubenchikova 1987). In the first aliquot the tissue was kept in a boiling water-bath for 20 min to facilitate the extraction of the pigment, the extracts were then filtered. Absorbance of the supernatant was measured at 525 nm using a Perkin Elmer spectrophotometer. The bulk of anthocyanin in the seedlings was in the cotyledons, but some was in the hypocotyls. In the experiment reported herein, total values are given. Chromatographic separations of anthocyanin extracts were carried out, with cyanidin as a reference, on paper

in water:glacial acetic acid:conc hydrochloric acid, 10:30:3 (by volume) (Bate-Smith 1954) and in n-butanol:2N hydrochloric acid, 1:1 (by volume) (Bate-Smith & Westall 1950). Unhydrolyzed 1% HCl extracts were chromatographed in n-butanol:glacial acetic acid:water, 12:3:5 by volume (Smith & Smith 1965).

Propanol, kinetin and EDTA treatments.

Two day old dark grown seedlings were transferred to a fresh Petri dish containing 4 ml of 1% n-propanol, 0.2% Kinetin or 0.5 mM EDTA for 15 min before or after exposure to R or FR light followed by return to the original dish. Control seedlings were treated in the same way using H_2O. A similar procedure was adopted for the $CaCl_2$ (7 mM) and cholesterol (0.1 mM) treatments but the seedlings were kept in the reagents for 48 hours (Pecket & Hathout 1974a).

Extraction of calcium:

Eighty seedlings were immersed in 20 ml EDTA (0.5 mM) or water for 15 minutes. The eluate was evaporated to dryness and the residue dissolved in 20 ml H_2O. To 1 ml, an equal volume of 6.5% lanthanum was added and the extracted Ca^{+2} content assayed with a Perkin Elmer atomic absorption spectrophotometer at 422 nm. A calibration curve of Ca^{+2} in water was also carried out.

RESULTS AND DISCUSSION

In preliminary work it was established that the biosynthesis of anthocyanin in dark grown seedlings of turnip is unaffected by treatment with $CaCl_2$ over the range 0-10 mg/l. However, the application of $CaCl_2$ to two day old dark grown seedlings following a five minute exposure to red light nullifies the effect of the light treatment (Table 1). These results are consistent with the view that calcium has a stabilizing effect on a membrane which is involved in the regulation of the passage of substrate(s) of anthocyanin biosynthesis in red cabbage to the enzymes involved in the synthesis (Pecket & Hathout 1974b).

The stimulation of dark synthesis by propanol and by kinetin is markedly reduced by subsequent treatment with $CaCl_2$. Cholesterol also reduces the stimulatory effects of R light, propanol or kinetin (Table 1).

Seedlings were grown for two days in darkness and then treated for five min with R light, 1% PrOH or 0.2% kinetin for 15 min. Immediately after these treatments, $CaCl_2$ (7 mM) or cholesterol (0.1 mM) was applied to the seedlings for a further period of 48 hr in darkness. The figures are means of eight observations and standard errors are shown.

If the effect of applied calcium is to stabilize the membrane and thereby prevent the increase in permeability induced by such factors as R light, then

Table 1: Effect of $CaCl_2$ and cholesterol on anthocyanin synthesis in turnip seedlings.

	Anthocyanin content (525 nm)			
Treatment	Dark	R	PrOH	Kinetin
Water	0.32±0.02	0.47±0.02	0.65±0.02	0.49±0.02
$CaCl_2$	0.30±0.02	0.31±0.03	0.39±0.03	0.32±0.02
Cholesterol	0.35±0.02	0.35±0.02	0.36±0.02	0.34±0.03

it is possible that native calcium in the membrane may itself be involved in the normal regulation of the passage of substrates. In order to investigate this possibility, the influence upon anthocyanin synthesis of EDTA (which chelates Ca^{-2} [Burstrom 1968; Foote & Hanson 1964]) was investigated. Seedlings were treated with EDTA for 15 min before or after five min exposure to R or FR light. EDTA stimulates anthocyanin synthesis in dark grown seedlings (Figure 1). When applied either before or after R light treatment, EDTA does not appear to promote a greater stimulation than R light on its own. However, when FR exposure is employed instead of R, the effect depends upon the sequence of the treatments. Application of EDTA promotes synthesis when applied before the FR exposure but not when the sequence is reversed (Figure 1). These results suggest that FR light may in some way render native calcium less readily chelatable by EDTA. However, if the EDTA treatment precedes the FR exposure, insufficient free Ca^{+2} is present to stabilize the membrane.

Anthocyanin synthesis in the seedlings takes place in the epidermal and sub-epidermal cells of the cotyledons and hypocotyl (Pecket & Small 1980). Chromatographic examination revealed the same major anthocyanin in cotyledons and hypocotyl of turnip seedlings. This was a glycoside of cyanidin. Harborne (1963) reported the pigment of red cabbage to be cyanidin-5-glucoside-3-sophoroside (in acylated form). The principal site of synthesis is the cotyledons, and it would therefore appear likely that the effects of EDTA reported above arise following the uptake and translocation of this substance to the sites of anthocyanin synthesis. In an attempt to ascertain whether this explanation, concerning the binding of Ca^{+2} might be valid, assays of extractable Ca^{+2} were carried out on solutions in which seedlings had been completely immersed for 15 minutes after a five minute exposure to R or FR light. When water was used for extraction, only small quantities of Ca^{+2} were removed and no significant differences were found between the treatments (Figure 2).

However, when EDTA was employed as extractant, a substantially larger amount of Ca^{+2} was removed and there was a highly significant increase in extractable Ca^{-2} from seedlings which had received a prior exposure to five minutes R light (Figure 2). Assays of extractable Ca^{-2} from separated cotyledons and hypocotyls revealed that approximately twice as much Ca^{+2} came

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Fig (1) Effect of EDTA on phytochrome controlled anthocyanin synthesis in turnip.

Fig (2) Effect of R, FR and darkness on extractable Ca^{2+} from seedlings of turnip.

from the former organs, the principal site of anthocyanin synthesis. These observations suggest that the photocontrol of anthocyanin biosynthesis may involve changes in permeability which result from alterations in the capacity to bind Ca^{+2} into a membrane. It seems possible that the extent to which Ca^{+2} is bound into a membrane and thereby stabilizes the membrane, is a function of the molecular configuration of the phytochrome molecule. If this is so, then the Pr form of phytochrome must have a greater capacity to bind Ca^{+2} than the Pfr form. It is generally assumed that Pfr is the active form of phytochrome in respect to the promotion of photomorphogenetic events (Hendricks & Borthwick 1967). The interpretation placed upon this data implies that the activity of Pfr may result from its being less able to bind Ca^{+2} in membrane(s). The increase in permeability which would result would lead to the freer movement of substances of importance in plant development through the membranes. The evidence presented herein is consistent with Pr exercising a restraint upon the movement of substrates for anthocyanin biosynthesis by virtue of its greater ability to bind Ca^{+2}.

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